

## **REMARKS**

By this amendment, Applicants have made minor amendments to Claims 46, 59 and 70. Claims 57, 67, 71-81 and 88-90 were previously withdrawn from consideration, and Claims 16-56, 58-66, 68-70, 82-87 and 91 are under examination. For the reasons as set forth below, Applicants submit that the present application overcomes all prior rejections and has been placed in condition for allowance.

As an initial matter, Applicants have amended Claims 46, 59 and 70 to specify that the virus-like particles contain functional hepatitis C virus glycoproteins assembled onto retroviral core particles. This subject matter is supported in the original application, e.g., at page 14, lines 25-27, and no new matter has been added.

In the Official Action, the rejected Claims 46-56, 58-66, 68-70, 82-87 and 91 under 35 U.S.C. § 103(a) as being unpatentable over Marasco et al. PCT publication WO 00/55335, in view of both the Lechmann et al. 2001 article and the Ray et al. 2001 article. This rejection is respectfully traversed for the reasons that follow.

In the Official Action, the Examiner rejected the arguments filed in response to the prior action on the basis that the documents supporting these arguments only related to pseudotyped VSVs whereas the present invention would pertain to obtaining hepatitis C virus particles and not to obtaining pseudotyped VSV particles. According to the Examiner, producing these two types of particles would not present the same constraints: producing pseudotyped VSV particles would require the expression of E1 and E2 proteins at the cell surface, whereas producing HCV pseudoparticles according to the invention would

require the retention of E1 and E2 proteins in the endoplasmic reticulum. The Examiner's arguments are respectfully traversed for the reasons that follow.

Contrary to the Examiner's position, the HCV pseudoparticles according to the invention are pseudotyped particles and are therefore closer to pseudotyped VSV particles described for example in Matura et al. than to the "HCV-like particles" described in Ezelle et al. or Lechman et al. Moreover, the particles called "HCV-like particles" in Ezelle et al. correspond to an assembly of HCV core, E1 and E2 proteins which does not comprise heterologous VSV structural proteins (see page 12328). Similarly, the "HCV-like particles" described in Lechman et al. are not pseudoparticles in which the HCV envelope proteins are assembled on a retroviral core.

To the contrary, the HCV pseudoparticles defined in the amended set of claims contain structural proteins from a virus which is not HCV, on which functional HCV glycoproteins are assembled. Typical HCV pseudoparticles according to the invention comprise a retroviral Gag-Pol expressing HCV E1 E2 glycoproteins at their surface as exemplified in De Beeck et al. (2004) *J. Virol.* 78:2994-3002 and Sandrin et al. (2005) *J. Gen. Virol.* 86:3189-3199 (copies attached herewith).

Accordingly, the method of producing HCV pseudoparticles according to the invention is closer to the method of producing pseudotyped VSV particles described for example in Matura et al. than to the method of producing "HCV-like particles" described in Ezelle et al. or Lechman et al .

In the amended set of claims, the C-terminal transmembrane domain of the E1 and E2 proteins, encoded by the third nucleic acid sequence, is unmodified with respect to native E1 and E2 proteins. This feature is particularly important since it enables the pseudoparticles to become perfectly functional to enter into the cell.

Applicants submit that none of the cited documents, alone or in combination, teaches or suggests the use of such proteins with an unmodified transmembrane domain to produce hepatitis-like particles.

As indicated in the present application, e.g., at page 2, lines 8-11, pseudotyped VSV viral particles of the prior art were obtained with chimeric E1 and E2 glycoproteins, the transmembrane domains of which were modified to enable their trafficking to the cell surface. Matsuura et al. describes the production of HCV-VSV pseudotypes possessing chimeric HCV E1 or E2 glycoproteins. These chimeric E1 and E2 glycoproteins are characterized by the presence of ectodomains of E1 or E2 joined to the transmembrane and cytoplasmic domains of the VSV-G protein. The authors justify this modification by the fact that HCV E1 and E2 proteins bear, in their C-terminal portion, retention signals, which would prevent their expression at the cell surface (page 264, left column, § 2).

Similarly, Buonocore et al. describes VSV virions which are pseudotyped with the extracellular domain of HCV E1 and E2 proteins in fusion with the transmembrane and cytoplasmic domains of VSV G protein, in order to enable the expression of E1 and E2 proteins at the cell surface.

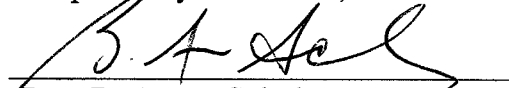
Accordingly, one skilled in the art would not have been motivated in view of these documents to combine Marasco et al. and Lechman et al. and thus to pseudotype viruses with HCV E1 and E2 proteins bearing an unmodified transmembrane domain. To the contrary, these documents **teach away** from the claimed invention and would have led one skilled in the art to consider that such pseudotyping was only possible with modified E1 and E2 devoid of retention signal.

Applicants thus submit that the present claims are not anticipated nor made obvious by the cited prior art of record, and that the Examiner's rejection on the basis of these references is respectfully traversed and should be withdrawn.

In light of the amendments and arguments provided herewith, Applicants submit that the present application overcomes all prior rejections and objections, and has been placed in condition for immediate allowance. Such action is respectfully requested.

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Respectfully submitted,



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## Characterization of Functional Hepatitis C Virus Envelope Glycoproteins

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Hepatitis C virus (HCV) encodes two envelope glycoproteins, E1 and E2, that assemble as a noncovalent heterodimer which is mainly retained in the endoplasmic reticulum. Because assembly into particles and secretion from the cell lead to structural changes in viral envelope proteins, characterization of the proteins associated with the virion is necessary in order to better understand how they mature to be functional in virus entry. There is currently no efficient and reliable cell culture system to amplify HCV, and the envelope glycoproteins associated with the virion have therefore not been characterized yet. Recently, infectious pseudotype particles that are assembled by displaying unmodified HCV envelope glycoproteins on retroviral core particles have been successfully generated. Because HCV pseudotype particles contain fully functional envelope glycoproteins, these envelope proteins, or at least a fraction of them, should be in a mature conformation similar to that on the native HCV particles. In this study, we used conformation-dependent monoclonal antibodies to characterize the envelope glycoproteins associated with HCV pseudotype particles. We showed that the functional unit is a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. We did not observe any evidence of maturation by a cellular endoprotease during the transport of these envelope glycoproteins through the secretory pathway. These envelope glycoproteins were recognized by a panel of conformation-dependent monoclonal antibodies as well as by CD81, a molecule involved in HCV entry. The functional envelope glycoproteins associated with HCV pseudotype particles were also shown to be sensitive to low-pH treatment. Such conformational changes are likely necessary to initiate fusion.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (39). This small enveloped positive-strand RNA virus has been classified within its own genus, *Hepacivirus*, within the *Flaviviridae* family, which also comprises the *Flavivirus* and *Pestivirus* genera (54). Its genome encodes a single polyprotein precursor of just over 3,000 amino acid residues. This polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield at least 10 polypeptides (36, 41). The two viral envelope glycoproteins, E1 and E2, are released from the HCV polyprotein precursor after cleavage by a host signal peptidase(s) (reviewed in reference 20).

No efficient and reliable cell culture system is available to amplify HCV (36). The current knowledge on the characterization of HCV envelope glycoproteins is based on cell culture transient-expression assays. HCV glycoproteins are type I transmembrane proteins with a large N-terminal ectodomain and a C-terminal hydrophobic anchor. During their synthesis, the ectodomains of HCV glycoproteins are targeted to the endoplasmic reticulum (ER) lumen, where they are modified by N-linked glycosylation. E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively (27). HCV envelope glycoproteins have been shown to assemble into oligomeric

complexes. They can form a heterodimer of E1 and E2 stabilized by noncovalent interactions as well as heterogeneous disulfide-linked aggregates (19). Extensive characterization of the noncovalent heterodimer strongly suggests that this oligomer is the prebudding form of the functional complex, which will probably subsequently play an active role in the process of entry into host cells (15).

Immunolocalization studies and glycan analyses have shown that the noncovalent E1E2 heterodimer is located in the ER (15, 22). In addition, the transmembrane domains of E1 and E2 have been shown to play a major role in ER retention of the E1E2 complex (10, 12, 14). Several attempts have been made to mutate the transmembrane domains of HCV envelope glycoproteins in order to readdress them to the plasma membrane with the objective of making pseudotyped viruses or developing a cell-cell fusion assay (6, 26, 40, 43, 51, 57, 59). However, the transmembrane domains of HCV envelope glycoproteins also play a role in heterodimerization (46), and this function is lost when these transmembrane domains are replaced by those of other proteins (12, 40). In addition, such mutations also abolish the entry functions of HCV envelope glycoproteins (31).

Recently, infectious pseudotype particles that are assembled by displaying unmodified HCV envelope glycoproteins on retroviral core particles have been successfully generated (4, 31). The data that have been accumulated on these pseudotype particles strongly suggest that they mimic the early steps of HCV infection. Indeed, they exhibit a preferential tropism for hepatic cells, and they are specifically neutralized by anti-E2

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monoclonal antibodies (MAbs) as well as sera of HCV-infected patients. These HCV pseudotype particles (HCVpp) therefore represent the best tool currently available to study functional HCV envelope glycoproteins. In this report, we characterized HCVpp-associated envelope proteins by use of conformation-dependent MAbs. We showed that the functional unit is a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. We did not observe any evidence of a maturation by cellular endoprotease cleavage during their transport through the secretory pathway. In addition, conformational changes in HCV envelope glycoproteins were observed after low-pH treatment.

#### MATERIALS AND METHODS

**Cell culture.** Huh-7 human hepatocellular carcinoma cells (45) and 293T human embryo kidney cells (293tsA1609neo) obtained from the American Type Culture Collection (Manassas, Va.) were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

**Production of HCVpp and infection assays.** Production of HCVpp and infection assays have been described previously (4). In this work, we used the cytomegalovirus (CMV)-Gag-Pol murine leukemia virus (MLV) packaging construct, containing the MLV *gag* and *pol* genes and the MLV-green fluorescent protein (GFP) (4) or MLV-Luc (C. Voisset and J. Dubuisson, unpublished data) plasmid, encoding an MLV-based transfer vector containing a CMV-GFP or CMV-Luc internal transcriptional unit. Due to better sensitivity, the MLV-Luc plasmid was used for the neutralization assays. The HCV sequence used in this work is derived from the H strain (genotype 1a) (23). In some experiments, modified HCV envelope proteins were used. Plasmids pTM1/E1†HA and pTM1/E2†HA (13) were used to construct pHCMV-E1-HA and pHCMV-E2-HA, respectively. In these plasmids, the last residue (Ala) at the C terminus of E1 and E2 has been replaced by an Arg to avoid signal sequence cleavage. In several experiments, 293T cells were metabolically labeled from 16 to 40 h posttransfection with <sup>35</sup>S-Protein Labeling Mix (Amersham Biosciences) as described previously (19).

**Antibodies.** The MAbs used in this work have been described previously: A4 (19); H2 (15); H14 (8); H31, H33, H35, H44, H47, H48, H50, H52, H53, H54, H57, H60, and H61 (24, 25, 48); 1/39, 3/11, 6/16, 6/53, and 9/86a (25); CBH2, CBH4B, CBH4D, CBH4G, CBH5, CBH7, CBH11, and CBH17 (28). The rat anti-hemagglutinin (anti-HA) MAb (3F10) was purchased from Roche.

**Immunoprecipitation and endoglycosidase digestions.** Metabolically labeled 293T cells and HCVpp obtained from cell culture supernatants were lysed with 0.5% Igepal CA-630 in TBS (50 mM Tris-HCl [pH 7.5]–150 mM NaCl). Immunoprecipitations were carried out as described previously (21). In some experiments, HCVpp were acidified with 150 mM morpholineethanesulfonic acid (MES) at 37°C for 20 min at pH 5.5. The pH was back-neutralized with 150 mM triethanolamine (pH 7.5) before immunoprecipitation. For endoglycosidase digestion, immunoprecipitated proteins were eluted from protein A-Sepharose in 30  $\mu$ l of dissociation buffer (0.5% sodium dodecyl sulfate [SDS] and 1% 2-mercaptoethanol) by boiling for 10 min. The protein samples were then divided into three equal portions: one for digestion with endo- $\beta$ -N-acetylglucosaminidase H (endo H), one for digestion with peptide:N-glycosidase F (PNGase F), and one for an undigested control. Digestions were carried out for 1 h at 37°C in the buffer provided by the manufacturer (New England Biolabs). Digested samples were mixed with an equal volume of 2 $\times$  Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Western blotting.** Proteins bound to nitrocellulose membranes were revealed by enhanced chemiluminescence detection as recommended by the manufacturer (NEN). Briefly, after separation by SDS-PAGE under reducing conditions, proteins were transferred to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad) and revealed with specific MAbs (A4, 3/11, H52, or 9/27) followed by rabbit anti-mouse (A4 and H52) or anti-rat (3/11 and 9/27) immunoglobulin conjugated to peroxidase (dilution, 1/1,000; Dako).

**CD81 pull-down assay.** Recombinant fusion proteins containing the large extracellular loop of human or murine CD81 fused to glutathione *S*-transferase were preadsorbed onto glutathione-Sepharose 4B beads according to the manufacturer's recommendations (Pharmacia Biotech, Uppsala, Sweden). Pull-down experiments were performed as described previously (11). Precipitates were separated by SDS-PAGE followed by Western blotting with an anti-E1 (A4) or anti-E2 (3/11) MAb.

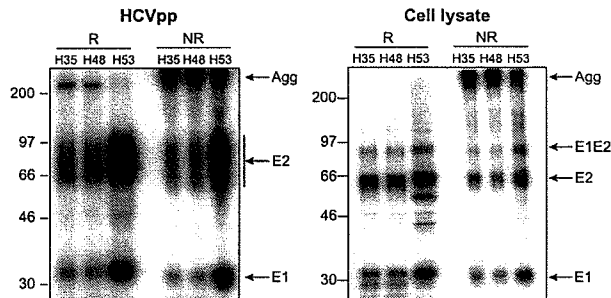


FIG. 1. Noncovalent E1E2 heterodimers are incorporated into HCVpp. At 16 h posttransfection, 293T cells transfected to produce HCVpp were metabolically labeled for 24 h. The supernatant (HCVpp) and cell lysate were immunoprecipitated with anti-E2 MAbs (H35, H48, and H53). The immunoprecipitates were analyzed by SDS-PAGE under reducing (R) or nonreducing (NR) conditions. Sizes of protein molecular mass markers are given on the left (in kilodaltons). HCV envelope proteins E1 and E2 as well as aggregates (Agg) and SDS-resistant E1E2 complexes are indicated on the right.

#### RESULTS

**Noncovalent E1E2 heterodimers are associated with HCVpp.** Extensive characterization of HCV envelope glycoproteins has shown that these proteins assemble as a noncovalent heterodimer which is most likely the prebudding form of the functional complex (15). Because assembly into particles and secretion from the cells lead to structural changes in viral envelope proteins, we were interested in analyzing the modifications associated with HCV envelope glycoproteins incorporated into HCVpp. Although we cannot exclude some heterogeneity in HCV proteins incorporated into HCVpp, we focused our work on proteins isolated with the help of conformation-dependent MAbs, some of which have been shown to have a neutralizing activity against HCVpp.

Conformation-dependent anti-E2 MAbs (H35, H48, and H53) were used to characterize HCV envelope glycoproteins incorporated into HCVpp. MAbs H35 and H48 have been shown to have a neutralizing activity when incubated with HCVpp (4), indicating that these antibodies recognize functional HCV envelope glycoproteins. As shown in Fig. 1 (HCVpp), two bands corresponding to E1 and E2 were detected after immunoprecipitation with MAbs H35, H48, and H53. Since these MAbs recognize an epitope present on E2, these results indicate that hetero-oligomers of HCV envelope glycoproteins are incorporated into HCVpp (Fig. 1). Similar results were obtained when infectious HCVpp were purified on a sucrose density gradient (data not shown). It is worth noting that HCVpp-associated E2 had a slower and more diffuse migration pattern than the cell-associated form. This is likely due to modifications of the glycans by Golgi enzymes (see below). When analyzed under nonreducing conditions, the same bands corresponding to E1 and E2 were still observed, indicating that HCV envelope glycoproteins formed noncovalent complexes involving E1 and E2. As observed before (42), some aggregates were observed on the top of the gel, but they represented a minor fraction of HCV envelope glycoproteins. Indeed, the intensities of the bands corresponding to E1 and E2 were very similar when analyzed under reducing and non-reducing conditions. Although the majority of E1 and E2 en-

velope glycoproteins associated with HCVpp form noncovalent heterodimers, we cannot exclude the possibility that the disulfide-linked E1E2 complexes play a role in HCV entry. When analyzed under reducing conditions, two glycoforms of E1 were immunoprecipitated from the cell lysate (Fig. 1, Cell lysate), as previously observed (18). Interestingly, only the fully glycosylated form of E1 was associated with HCVpp (Fig. 1, HCVpp). HCV glycoprotein complexes containing the lower-glycosylation form of E1 are therefore not incorporated into HCVpp. This finding is in agreement with the observation that this lower glycosylated form of E1 was associated with aggregates in the cell lysate, as it was detected only under reducing conditions (Fig. 1, Cell lysate).

Altogether, these results indicate that HCV envelope glycoproteins are associated with HCVpp as a noncovalent heterodimer.

**Maturation of the glycans associated with HCV envelope glycoproteins.** HCV envelope glycoproteins are modified by N-linked glycosylation. Analyses of the glycans bound to the intracellular HCV envelope glycoprotein heterodimer have indicated that only high-mannose type oligosaccharides are associated with these proteins (15, 22). The absence of modification of the glycans associated with HCV envelope glycoproteins indicates that they are not modified by Golgi enzymes, which is in agreement with their localization in the ER or an ER-like compartment.

To determine whether the glycans associated with HCV envelope glycoproteins expressed in 293T cell are modified by Golgi enzymes, their resistance to endo H treatment was determined. Indeed, endo H removes the chitobiose core of high-mannose oligosaccharides and some hybrid forms of N-linked sugars but not the complex forms (53). Hence, resistance to digestion with endo H is indicative that glycoproteins have moved from the ER to at least the medial and *trans*-Golgi regions, where complex sugars are formed. PNGase F treatment, which removes all types of N-linked glycans, was used as a control of deglycosylation. Since H35 and H48 had a lower relative affinity, we used MAb H53 to analyze the glycans associated with E1 and E2. Although H53 is not neutralizing, the proteins precipitated by this antibody have a pattern similar to those precipitated by MAbs H35 and H48 (Fig. 1). As shown in Fig. 2A (Cell lysate), cell-associated HCV envelope glycoproteins remained endo H sensitive in a steady-state labeling experiment, suggesting that the bulk of E1 and E2 expressed in 293T cells is located in an early compartment of the secretory pathway, as previously shown in other cell lines (15). As previously observed (19), the PNGase F-treated E2 migrated slightly faster than the endo H-treated protein, and the PNGase F-treated E1 had a slightly slower migration profile than the endo H-treated form (Fig. 2A, Cell lysate). In the case of E2, this could be explained by the presence of a residual *N*-acetylglucosamine at each glycosylation position after endo H treatment. The slightly slower migration of PNGase F-treated E1 is likely due to abnormal behavior of this protein after modification of its hydrophilicity as observed in C-terminally truncated forms (44).

To determine whether the glycans associated with HCV envelope glycoproteins incorporated into HCVpp are resistant to endo H treatment, metabolically labeled HCV envelope glycoproteins associated with HCVpp were immunoprecipitated

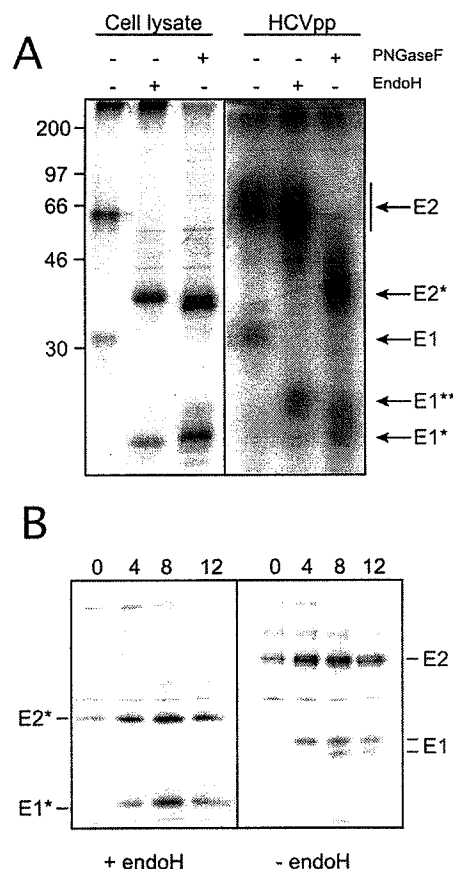


FIG. 2. Analyses of the glycans associated with HCV envelope proteins. (A) The glycans associated with HCV envelope glycoproteins are modified by Golgi enzymes. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants (HCVpp) and cell lysates were immunoprecipitated with the anti-E2 MAb H53. The immunoprecipitates were either left untreated or treated with endo H or PNGase F and analyzed by SDS-PAGE. Deglycosylated forms of E1 and E2 are indicated by an asterisk. The endo H-resistant form of E1 is indicated by two asterisks. Sizes of protein molecular mass markers are given on the left (in kilodaltons). (B) The bulk of HCV envelope glycoproteins expressed in 293T cells is not modified by Golgi enzymes. 293T cells transfected for 24 h were pulse-labeled for 30 min and chased for different times as indicated (in hours). Cell lysates were immunoprecipitated with the anti-E2 MAb H53. The immunoprecipitates were either left untreated or treated with endo H and analyzed by SDS-PAGE. HCV envelope proteins E1 and E2 are indicated on the right. Deglycosylated forms of E1 and E2 are indicated by an asterisk.

tated with MAb H53 and either left untreated or treated with endo H or PNGase F. As shown in Fig. 2A, HCVpp-associated E2 treated with endo H had a migration profile similar to that of the untreated protein. An additional faint band of 46 kDa was also detected, indicating some heterogeneity in the sensitivity to endo H. Together, these data indicate that most E2 glycans are endo H resistant. Interestingly, most HCVpp-associated E1 treated with endo H migrated faster than the untreated protein but more slowly than the deglycosylated form from the cell lysate (Fig. 2A; compare E1\*\* and E1\*). This indicates that at least one E1 glycan can be resistant to endo H treatment, while the others are sensitive and therefore likely of the high-mannose type.

PNGase F removes all types of N-linked glycans. However, after PNGase F treatment, two fast-migrating bands were detected for HCVpp-associated E1 (Fig. 2A). One migrated at the size of the fully deglycosylated protein, and the other had a slightly slower mobility, suggesting that this form still contained at least one glycan. Such a partial resistance to PNGase F treatment has already been observed for truncated forms of HCV glycoprotein E2 (J. Dubuisson, unpublished data) as well as for other proteins (30). Whether the PNGase F-resistant glycan of E1 is the same as the endo H-resistant glycan remains to be determined.

When HCV envelope proteins are overexpressed in 293T cells to produce HCVpp, a fraction of these proteins accumulate at the plasma membrane, the site where these particles have been suggested to bud (4, 16, 31). This suggests that HCV envelope glycoproteins that accumulate at the plasma membrane have followed the secretory pathway; thus, their glycans should be modified by Golgi enzymes. Because no endo H-resistant form of HCV envelope glycoproteins was detected in steady-state labeling, the sensitivity of HCV envelope glycoproteins to digestion with endo H was analyzed in a pulse-chase experiment, which is a more sensitive approach. As shown in Fig. 2B, HCV envelope proteins remained endo H sensitive, and even after 12 h of chase, no endo H-resistant form of E1 and/or E2 was clearly detected, suggesting that the fraction that migrates to the cell surface must be very small. This is in agreement with the presence of ER retention signals in HCV envelope glycoproteins (10, 12). Detection of E1 and/or E2 at the surfaces of 293T cells by immunofluorescence (4, 16, 31) is therefore likely due to small amounts of E1E2 proteins escaping the ER retention machinery.

Altogether, these results indicate that HCV envelope glycoproteins incorporated into HCVpp issue from a very small population of proteins escaping the ER retention machinery and are thus modified by Golgi enzymes and contain complex or hybrid type glycans.

**Maturation of HCV envelope glycoproteins.** Most viral envelope proteins are matured by cellular endoprotease cleavage during their transport through the secretory pathway (32). It was therefore interesting to determine whether such a maturation also occurred for HCV envelope glycoproteins. As shown in Fig. 1, SDS-PAGE analyses showed no evidence of a protease cleavage in E1 or E2. HCVpp-associated E2 migrated more slowly than the cell-associated form, and the migration profiles of E1 were very similar for the HCVpp- and cell-associated forms (Fig. 1). In addition, after deglycosylation by PNGase F treatment, neither E1 nor E2 from HCVpp migrated faster than the deglycosylated cell-associated forms (Fig. 2A). However, we could not exclude a cleavage that would not be detected by SDS-PAGE. Indeed, some C-terminally truncated forms of E1 have been shown to migrate more slowly than expected due to abnormal behavior in SDS-PAGE (44). Therefore, we could not totally rule out a cleavage very close to the N or C terminus of E1 or E2. To exclude an N-terminal cleavage, HCV envelope glycoproteins associated with HCVpp were analyzed by Western blotting with antibodies recognizing an epitope located at the N terminus of E1 or E2. If a cleavage has occurred, the peptide should be separated from the rest of the protein when analyzed by SDS-PAGE, and such antibodies should not recognize the bulk of the protein in

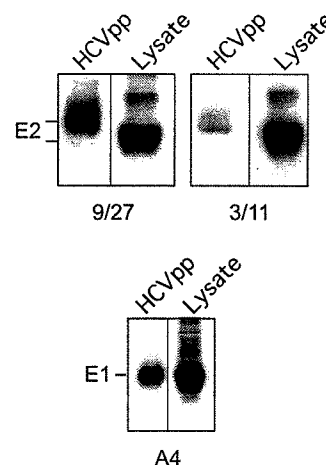


FIG. 3. Detection of the N termini of HCV envelope glycoproteins. HCVpp purified on a 20-to-60% sucrose gradient and lysates from cells expressing E1E2 were analyzed by Western blotting with MAbs recognizing an epitope located at the N terminus of E1 (MAb A4, recognizing amino acids 197 to 207) or E2 (MAb 9/27, recognizing amino acids 396 to 407). The anti-E2 MAb 3/11 was used as a control.

Western blotting. We used MAbs A4 and 9/27, which are directed against epitopes located at the N termini of E1 (amino acids 197 to 207) and E2 (amino acids 396 to 407), respectively. As shown in Fig. 3, an E2 protein of the appropriate size was detected by Western blotting with MAb 9/27, indicating that the N terminus of E2 had not been cleaved. As a control to confirm the absence of a partial cleavage, we used an antibody recognizing an epitope located elsewhere on E2 (MAb 3/11) and compared the amounts of protein recognized by the two antibodies. As shown in Fig. 3, there was no evidence of any relative decrease in the amount of E2 recognized by MAb 9/27, suggesting that no partial cleavage had occurred. Although 12 residues separate the N terminus of E2 from the epitope recognized by MAb 9/27, a cleavage within this 12-amino-acid hypervariable segment is unlikely. As shown in Fig. 3, an E1 protein of the appropriate size was also detected by Western blotting with MAb A4, indicating that the N terminus of E1 had not been cleaved. In the absence of an antibody recognizing an epitope located elsewhere on E1, we compared the E1/E2 ratios between cell lysates and purified pseudotyped particles to detect a partial cleavage. Similar ratios were observed (data not shown), suggesting that no partial cleavage had occurred at the N terminus of E1.

To exclude a C-terminal cleavage, HCV envelope glycoproteins were tagged with an HA epitope at their C termini, and HCV envelope glycoproteins associated with HCVpp were analyzed by immunoprecipitation with MAb H53 followed by Western blotting with the anti-HA antibody. Western blot analyses with anti-E1 and anti-E2 MAbs indicated that E1†HA and E2†HA were efficiently incorporated into pseudoparticles when these proteins were coexpressed with their wild-type partners (Fig. 4A). Similar results were obtained when HCVpp were purified on a sucrose density gradient (data not shown). The presence of the tag reduced the infectivity of the pseudoparticles to approximately 35% in the case of E2 and 4% in the case of E1 (Fig. 4B). Since the HA tag does not alter E1E2



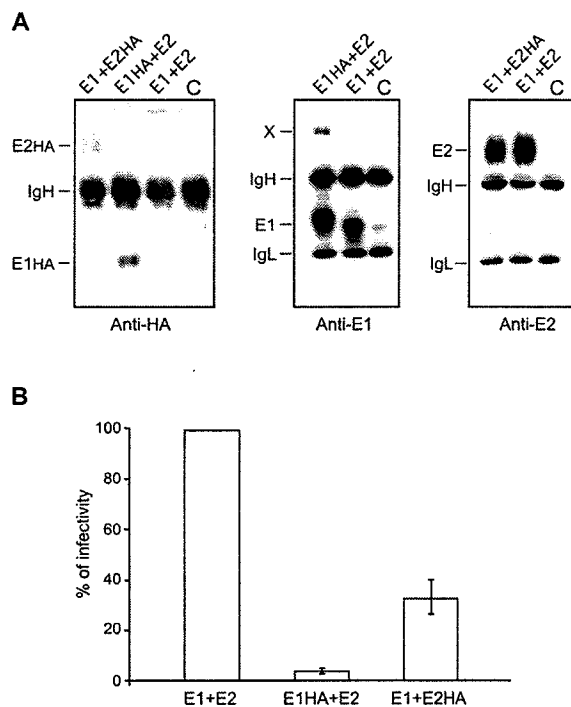


FIG. 4. Detection of the C termini of HCV envelope glycoproteins. (A) The envelope glycoproteins associated with HCVpp generated with either E1 plus E2, E1HA plus E2, or E1 plus E2HA or without envelope glycoproteins (control [C]) were analyzed by immunoprecipitation with MAb H53 followed by Western blotting with the anti-HA MAb 3F10, the anti-E1 MAb A4, or the anti-E2 MAb H52. Immunoglobulin light (IgL) and heavy (IgH) chains and SDS-resistant E1 oligomers (X) are indicated. (B) Infectivity of HCVpp containing HCV envelope proteins tagged at their C termini with an HA tag was evaluated by using the luciferase reporter gene. The infectivity of HCVpp containing E1HA plus E2 or E1 plus E2HA was compared to that of HCVpp containing wild-type HCV envelope glycoproteins (E1 plus E2).

assembly (13), it likely affects the function of the transmembrane domains of these proteins during the fusion process, as shown for some mutations introduced into the transmembrane domain of the G protein of vesicular stomatitis virus (VSV) (9). As shown in Fig. 4A, E1HA and E2HA were detected by Western blotting with the anti-HA antibody, indicating that these proteins had not been cleaved in their C-terminal regions.

Altogether, these results indicate that there is no evidence that HCV envelope glycoproteins incorporated into HCVpp are cleaved by a cellular endoprotease during their transport through the secretory pathway. However, we cannot totally exclude cleavage of a small proportion of HCV envelope proteins associated with HCVpp.

**Epitope exposure on mature E1E2 glycoproteins.** MAbs are potentially very useful tools for structure-function studies of HCV envelope proteins and for defining B-cell epitopes that might have some relevance for therapeutic and/or vaccine development. Large panels of MAbs recognizing HCV envelope glycoproteins have been generated. Since there is no efficient and reliable cell culture system to amplify HCV, these MAbs have been screened against recombinant HCV envelope gly-

coproteins for their selection, and most of them have also been generated by immunizing mice or rats with the same recombinant proteins. The relevance of these tools has therefore not been confirmed on proteins that are functional for virus entry. HCVpp make it possible for the first time to reanalyze such antibodies in the context of potentially fully functional HCV envelope glycoproteins.

The binding of a panel of anti-E2 MAbs that have been generated in several laboratories, including ours (8, 15, 24, 25, 28, 48), to HCV glycoproteins associated with HCVpp was analyzed by immunoprecipitation. Among these MAbs, 3/11, H35, and H48 have been shown to neutralize approximately 70% of HCVpp infectivity, and H54 and H60 have been shown to neutralize approximately 40% of HCVpp infectivity (4, 31). As shown in Fig. 5, all the conformation-dependent MAbs described previously (H2, H31, H33, H35, H44, H48, H50, H53, H54, H57, H60, H61, and 9/86a) recognized HCV envelope glycoproteins associated with HCVpp. MAb H14, which specifically recognizes aggregates of HCV envelope glycoproteins (8), did not immunoprecipitate HCVpp-associated proteins. Interestingly, a series of human MAbs (CBH2, CBH4B, CBH4D, CBH5, CBH7, and CBH17) also recognized HCV glycoproteins associated with HCVpp. It is worth noting that all the conformation-independent MAbs (H47, H52, 1/39, 3/11, 6/16, 6/53, and CBH17) recognized the envelope glycoproteins associated with HCVpp less efficiently than the majority of the conformation-dependent MAbs (H2, H31, H33, H35, H44, H48, H50, H53, H54, H57, H60, H61, CBH2, CBH4B, CBH4D, CBH5, and CBH7). This suggests that conformation-sensitive epitopes are better exposed on functional HCV envelope glycoproteins.

Since human MAbs have been obtained from B cells of HCV-infected patients, they represent antibodies that have been generated against native antigens in the course of a normal HCV infection. In the absence of an efficient cell culture system to amplify HCV, these antibodies therefore represent valuable tools for the characterization of HCV envelope glycoproteins. To further characterize these antibodies, we analyzed their capacities to neutralize HCVpp. As shown in Fig. 6, two antibodies (CBH5 and CBH7) significantly reduced the infectivity of HCVpp. The incompleteness of the neutralization is likely due to the heterogeneity of E2 glycosylation. Indeed, due to the high level of glycosylation of E2 (27), some modified glycans might potentially partially mask epitopes. However, the observation that CBH5 and CBH7 have a neutralizing activity provides additional support for the observation that HCVpp mimic the early steps of infection of native HCV.

Enveloped viruses enter cells through two main pathways: direct fusion at the plasma membrane and receptor-mediated endocytosis. In the latter case, the fusion of the viral envelope protein(s) is triggered by low pH within the endosome. It has been shown that the infectivity of HCVpp is pH dependent (5, 31). This suggests that changes in the conformation of HCV envelope proteins occur at low pH. We were thus interested in seeing whether such changes would be detectable with our MAbs. HCV envelope glycoproteins associated with HCVpp were exposed to a low pH before immunoprecipitation with our panel of anti-E2 MAbs. As shown in Fig. 5, some changes were observed. For most antibodies, there was a slight reduction in the signal of E2 after low-pH treatment. Interestingly,

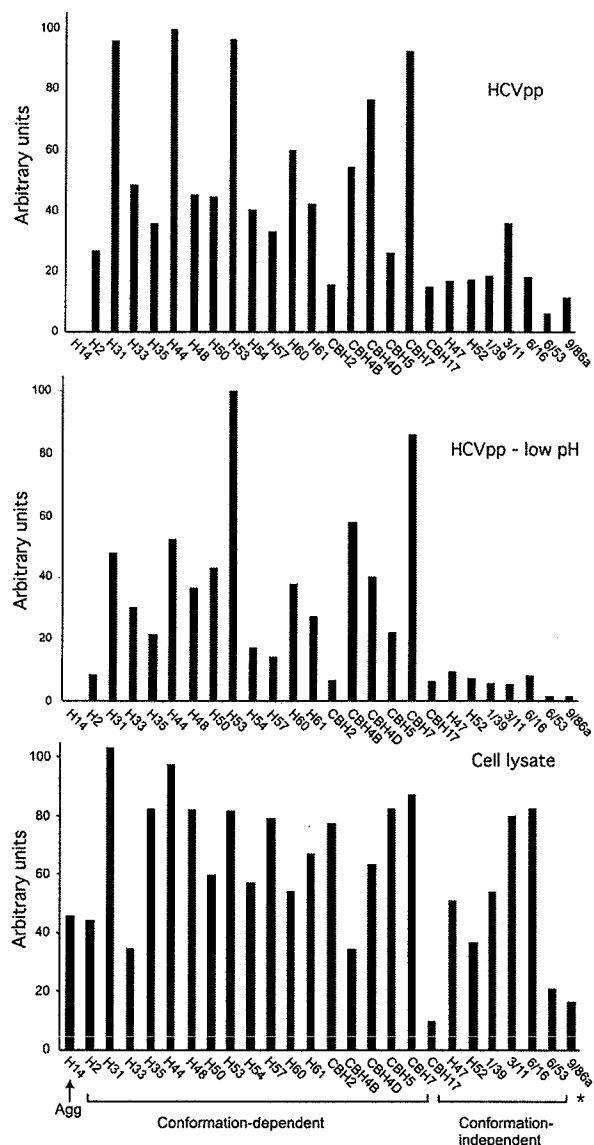


FIG. 5. Epitope exposure on HCV glycoproteins associated with HCVpp. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants (HCVpp) and cell lysates were immunoprecipitated with anti-E2 MAbs. In some experiments, supernatants were treated with MES (pH 5.5) for 20 min at 37°C (HCVpp-low pH) and pH was neutralized before immunoprecipitation. The intensities of immunoprecipitated E2 were determined by phosphorimaging. MAbs are grouped as conformation dependent or conformation independent. The epitopes of the following antibodies have been identified: 6/16 (amino acids 384 to 395), 9/86a (amino acids 384 to 407), 3/11 (amino acids 412 to 423), 1/39 (amino acids 432 to 443), H47 (amino acids 448 to 463), 6/53 (amino acids 544 to 551), and H52 (amino acids 644 to 655). Although it recognizes HVR1, MAb 9/86a (asterisked at the bottom) is conformation dependent. H14 (Agg) recognizes aggregates of HCV envelope glycoproteins.

the signal was almost totally abolished for a series of conformation-independent MAbs (1/39, 3/11, 6/16, 6/53, and 9/86a). In addition to changes in the recognition of E2 epitopes, the low-pH treatment also induced some dissociation of E1E2 complex. As shown for MAb H50 as an example, the amount of E1 that coprecipitated with E2 was reduced after low-pH

treatment (Fig. 7). The intensities of the bands were measured with a PhosphorImager, and the quantitative analysis of the bands indicated that approximately 75% of E1E2 complexes were dissociated. Together, these data indicate that conformational changes occur in E1E2 at low pH.

**E1E2 heterodimer associated with HCVpp interacts with CD81.** Human CD81 has been repeatedly shown to interact with recombinant soluble E2, E1E2 complex, HCV-like particles, and HCV particles from infectious plasma (7, 17, 25, 28, 34, 47, 50, 55, 60, 63). For the characterization of functional HCV envelope glycoproteins, it is therefore important to analyze their interaction with CD81. A CD81 pull-down assay was performed for this purpose. As shown in Fig. 8, both E1 and E2 from HCVpp were pulled down with human CD81 but not with the murine protein. These data confirm that the E1E2 heterodimer associated with HCVpp interacts with human CD81, which is in agreement with CD81 playing a role in HCV entry (4, 31).

## DISCUSSION

HCV envelope glycoproteins in their mature form, as they are present at the surface of the particle, have never been characterized. Because HCVpp contain fully functional envelope glycoproteins, these envelope proteins, or at least a fraction of them, are supposed to be in a mature conformation similar to that present on native HCV particles. HCVpp therefore represent the best tool currently available to characterize functional HCV envelope glycoproteins. Here, we characterized HCVpp-associated envelope proteins with conformation-

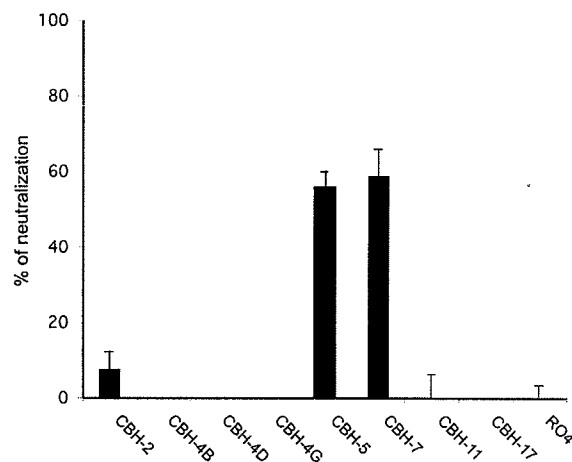


FIG. 6. Neutralization of HCVpp by human MAbs. HCVpp were preincubated, before infection of Huh-7 cells, with saturating concentrations (20 µg/ml) of human anti-E2 MAbs. The saturating concentrations were determined by analyzing the extent of neutralization of HCVpp with several concentrations of several neutralizing antibodies (data not shown). A negative-control experiment using a nonspecific human MAb (RO4) was performed. Control pseudotype particles produced in the absence of envelope proteins were used to establish the background level, which was always below 1% (data not shown). Pseudotype particles bearing VSV-G protein were used in a control neutralization experiment. No neutralization response was observed with these control particles (data not shown). Results, determined by measuring the luciferase activity, are expressed as percentages of neutralization.

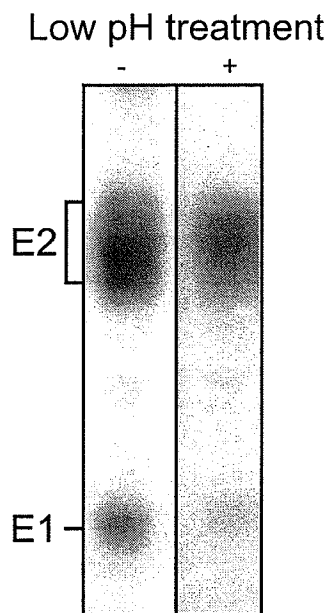


FIG. 7. Low-pH treatment dissociates E1E2 complexes. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants, either left untreated or exposed to a low pH, were immunoprecipitated with the anti-E2 MAb H50. Immunoprecipitates were analyzed by SDS-PAGE.

dependent MAbs, some of which had been shown to have neutralizing activity against HCVpp. We showed that these HCV envelope glycoproteins associated with HCVpp formed a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. In contrast to what has been observed for most viral envelope proteins, we did not detect any cleavage by a cellular endoprotease during their transport through the secretory pathway. In addition, HCV envelope glycoproteins were recognized by a large panel of MAbs and were shown to interact with CD81 and to be sensitive to low-pH treatment.

Noncovalent E1E2 heterodimers are associated with HCVpp. From sedimentation analyses, there is currently no evidence that larger oligomers of E1E2 are formed (A. Op De Beeck, unpublished data). Interestingly, some of the MAbs used to characterize the E1E2 complex show neutralizing activity (4; this work), indicating that they recognize fully functional envelope glycoproteins. Earlier studies of HCV envelope glycoproteins analyzed in transient expression systems have shown that these proteins can form noncovalent E1E2 heterodimers as well as heterogeneous disulfide-linked aggregates (19, 52). Extensive characterization of the noncovalent heterodimer with conformation-dependent MAbs has strongly suggested that this oligomer is most likely the prebudding form of the functional complex (15). We now show that the mature HCV glycoproteins form a complex showing some similarities to the prebudding E1E2 heterodimer. Very recently, a MAb produced by immunizing mice with an HCV antigen obtained from a chronically infected plasma has been shown to recognize disulfide-bound complexes that might potentially be formed of E1 and E2 (49). However, there is no evidence that the HCV envelope glycoproteins recognized by this MAb are

associated with infectious particles, because there was no purification of viral particles either to generate the antibody or to analyze its reactivity. There is, therefore, no clear evidence that this MAb recognizes envelope glycoproteins that are functional in HCV entry.

HCV envelope glycoproteins E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively (27). It has been confirmed experimentally by site-directed mutagenesis that four of the five putative glycosylation sites of E1 of genotype 1a can be occupied by N-glycans (42). The glycosylation sites occupied in E2 have not been determined yet. However, expression of HCV glycoprotein E2 followed by deglycosylation indicates that a large number of glycosylation sites are occupied. Analyses of the glycans bound to the intracellular HCV envelope glycoprotein heterodimer have indicated that high-mannose type oligosaccharides are associated with these proteins (15, 22). A fraction of HCV envelope glycoproteins overexpressed in 293T cells has been shown to accumulate at the plasma membrane, where they are supposed to be incorporated into HCVpp (4, 16, 31). However, our results suggest that expression of E1 and E2 on the cell surface is likely due to the accumulation of small amounts of E1E2 proteins escaping the ER retention machinery. The envelope glycoproteins associated with HCVpp contain complex type glycans. These proteins have therefore been transported through the secretory pathway, and some of the N-linked glycans associated with them have been modified by Golgi enzymes. This is in agreement with lectin binding analyses of HCV particles isolated from infected patients which suggest that the envelope glycoproteins of HCV might contain complex type glycans (56). Although the glycans associated with E2 proteins incorporated into HCVpp are modified by Golgi enzymes, most E1 glycans remain high-mannose type glycans. Indeed, endo H treatment experiments suggest that E1 might have a single glycan of the

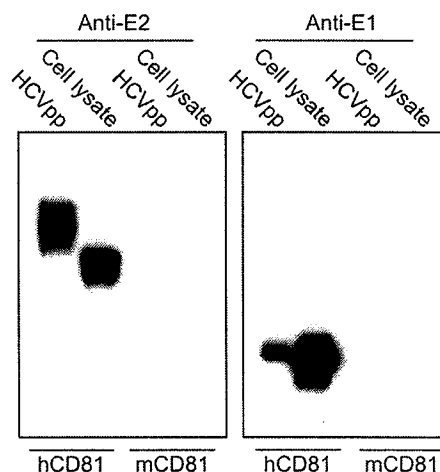


FIG. 8. E1E2 heterodimers associated with HCVpp interact with CD81. Supernatants (HCVpp) and cell lysates of 293T cells transfected to produce HCVpp were pulled down with a recombinant fusion protein containing the large extracellular loop of human CD81 (hCD81) or murine CD81 (mCD81) fused to glutathione *S*-transferase preadsorbed onto glutathione-Sepharose 4B beads. Precipitates were separated by SDS-PAGE followed by Western blot analysis with an anti-E1 (A4) or anti-E2 (3/11) MAb.

complex type. This conclusion contrasts with the previous observation that a C-terminally truncated form of E1 had all its glycans resistant to endo H treatment after secretion (44). These conflicting observations suggest that the presence of E2 masks the access of the Golgi enzymes to most E1 glycans. Alternatively, the truncated form of E1 might be more accessible to Golgi enzymes due to its misfolding. It has also been shown previously that different glycoforms of intracellular E1 can be observed (18). Here, we show that only the fully glycosylated form of E1 is incorporated into HCVpp.

The surface proteins of many enveloped viruses are initially synthesized as inactive precursors, and proteolytic cleavage is often required for maturation and full functional activity. In several virus families, this processing step is carried out by cellular proprotein convertases (32). In the case of the flaviviruses, the envelope contains two proteins, E and M. The latter is synthesized as a precursor called prM (29). Newly synthesized E and prM proteins associate to form heterodimers (2, 62) that are incorporated into immature virions by budding into the ER lumen (38). The particles are then transported through the secretory pathway, and shortly before release from the cell, they are converted to the active form by cleavage of prM by a cellular furin protease in the *trans*-Golgi network (58). Heterodimeric interactions between prM and E are important for proper folding of E (3, 33, 37) and probably also for protection of the immature virion against acid inactivation during transport through acidic vesicles (29). Such a maturation of the envelope glycoprotein complex is not observed for HCV. Whether modification of the glycans associated with HCV envelope proteins is responsible for the maturation of the fusion-competent E1E2 complex remains to be determined.

HCV envelope glycoproteins associated with HCVpp are sensitive to low-pH treatment. This finding is in agreement with the observation that HCVpp cell entry is pH dependent (5, 31). Conformational changes in viral fusion proteins are necessary for exposure of the fusion peptide, which interacts with the target membrane and thus initiates fusion. Interestingly, low-pH treatment induces dissociation of the E1E2 heterodimer. This is likely necessary to induce homo-oligomerization of the active form of the fusion protein, as shown for alphavirus envelope glycoproteins (61). There is, however, some controversy about the identity of the HCV fusion protein. It was first proposed that E1 might be a good candidate, because sequence analyses suggest that the ectodomain of E1 might contain a putative fusion peptide (26). However, potential structural homology with other fusion proteins from the same family, as well as with other type II fusion proteins, suggests that E2 should be the fusion protein (35, 64). Mutagenesis studies of the putative fusion peptides of the envelope glycoproteins associated with HCVpp, as described for the flavivirus envelope protein E (1), will be helpful for further characterization of the HCV fusion protein.

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# Assembly of functional hepatitis C virus glycoproteins on infectious pseudoparticles occurs intracellularly and requires concomitant incorporation of E1 and E2 glycoproteins

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Hepatitis C virus (HCV) E1 and E2 envelope glycoproteins (GPs) displayed on retroviral cores (HCVpp) are a powerful and highly versatile model system to investigate wild-type HCV entry. To further characterize this model system, the cellular site of HCVpp assembly and the respective roles of the HCV GPs in this process were investigated. By using a combination of biochemical methods with confocal and electron microscopic techniques, it was shown that, in cells producing HCVpp, both E1 and E2 colocalized with retroviral core proteins intracellularly, presumably in multivesicular bodies, but not at the cell surface. When E1 and E2 were expressed individually with retroviral core proteins, only E2 colocalized with and was incorporated on retroviral cores. Conversely, the colocalization of E1 with retroviral core proteins and its efficient incorporation occurred only upon co-expression of E2. Moreover, HCVpp infectivity correlated strictly with the presence of both E1 and E2 on retroviral cores. Altogether, these results confirm that the E1E2 heterodimer constitutes the prebudding form of functional HCV GPs and, more specifically, show that dimerization with E2 is a prerequisite for efficient E1 incorporation onto particles.

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## INTRODUCTION

Hepatitis C virus (HCV) has been classified within the genus *Hepacivirus*, family *Flaviviridae* (Robertson *et al.*, 1998), and is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Major *et al.*, 2001). The HCV genome encodes a single, approximately 3000 aa polyprotein precursor that is processed co- and post-translationally by cellular and viral proteases to yield at least 10 polypeptides, comprising structural components including the capsid protein and the envelope glycoproteins (GPs) E1 and E2, as well as non-structural proteins (Lindenbach & Rice, 2001; Penin *et al.*, 2004). HCV replicates its genome in a membrane-associated complex, which is derived from the endoplasmic reticulum (ER) and has been designated 'membraneous web' (Gosert *et al.*, 2003). All HCV polypeptides have been found associated with these membraneous web structures in a direct or indirect fashion (Dubuisson *et al.*, 2002; Egger *et al.*, 2002), suggesting that genome

replication occurs intracellularly within these structures (Egger *et al.*, 2002; Penin *et al.*, 2004). Morphogenesis of HCV is thought to occur within the secretory pathway, because particles containing HCV GPs have been detected in cytoplasmic vesicles of cells replicating the wild-type virus (Shimizu *et al.*, 1996). Furthermore, other viruses of the same family, including members of the genus *Flavivirus*, are thought to assemble intracellularly and bud into vesicles of the secretory pathway that are then released from the cell by exocytosis (Lindenbach & Rice, 2001; Mackenzie & Westaway, 2001).

The HCV envelope GPs expressed in heterologous systems were shown to be retained at the ER membrane by retention signals, including charged residues in the middle of their transmembrane domains (Cocquerel *et al.*, 1998, 1999; Flint & McKeating, 1999). Mutation of these charged residues has been shown to abolish ER retention, but also to interfere with heterodimerization of E1 and E2 (Cocquerel *et al.*, 2000; Michalak *et al.*, 1997; Op De Beeck *et al.*, 2000; Patel *et al.*, 2001). Indeed, the transmembrane domains of E1 and E2 play a major role in the assembly of E1 and E2 into

non-covalently attached heterodimers, which are thought to be the prebudding form of the HCV GPs (Deleersnyder *et al.*, 1997; Dubuisson, 2000).

Since its discovery 16 years ago, HCV has been difficult to study because it does not replicate efficiently or form particles *in vitro*. To establish surrogate model systems for HCV particle production, several laboratories initially tried to develop virus-like particles or pseudotype viruses by, for example, incorporating the HCV GPs onto cores of heterologous viruses, including those of vesicular stomatitis virus (VSV) or influenza virus (Flint *et al.*, 1999; Lagging *et al.*, 1998; Matsuura *et al.*, 2001; Takikawa *et al.*, 2000). In such systems, assembly was thought to take place at the cell surface; therefore, the HCV GPs were retargeted away from the ER to the plasma membrane by mutation or replacement of their transmembrane domains to achieve relocalization and thus incorporation onto heterologous viral cores. Because assembly and functionality of HCV GPs are very sensitive to mutations and deletion within their transmembrane domains and because the HCV GPs have a tendency to misfold and aggregate (Cocquerel *et al.*, 2000; Dubuisson, 2000; Dubuisson *et al.*, 2000), these attempts were mainly unsuccessful (Buonocore *et al.*, 2002). Recently, production methods for replication-competent HCV particles *in vitro* (HCVcc) have been reported; however, they are restricted to safety-level 3 laboratories (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005).

We and others have recently described HCV pseudoparticles (HCVpp) that are assembled by incorporating unmodified, full-length HCV GPs onto oncoretroviral or lentiviral cores (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Hsu *et al.*, 2003) that are highly infectious and that seem to mimic the viral entry and serological properties of wild-type HCV (Bartosch *et al.*, 2003a, c; Logvinoff *et al.*, 2004). HCVpp can be produced in large quantities at comparatively high titres and at a convenient safety level. Furthermore, they offer great flexibility in terms of incorporation of marker genes and allow investigation of viral entry independently of replication, as attachment and fusion are mediated by the HCV GPs and post-fusion steps are mediated by retro- or lentiviral core particles. Due to these features, HCVpp are likely to remain a valuable tool that will complement studies with the wild-type virus.

HCVpp are produced by expressing the E1E2 glycoproteins, the retroviral core proteins and a packaging-competent retroviral genome carrying a marker gene in human 293T cells (Bartosch *et al.*, 2003b). Viruses assembled by the 293T producer cells are collected from the supernatant and used to infect naive target cells. Within the 293T producer cells, E1 and E2 are expressed mainly at the ER, but a small fraction traffics to the cell surface (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Hsu *et al.*, 2003). Recent insights into retroviral assembly show that assembly and incorporation of diverse viral GPs may not take place at the cell surface, but can occur intracellularly within the endocytic pathway (Nydegger *et al.*, 2003; Pelchen-Matthews

*et al.*, 2003; Sherer *et al.*, 2003). In particular, budding of human immunodeficiency virus and *Murine leukemia virus* (MLV) has been shown to occur into multivesicular bodies (MVBs), a late endosomal compartment that can fuse with the cell surface (Gould *et al.*, 2003). We therefore investigated here the cellular site of assembly of MLV-based HCVpp. By using a combination of biochemical methods and confocal and electron microscopy (EM) to reveal the cellular localization of expressed E1 and E2 GPs, as well as MLV core proteins, we show that HCVpp bud intracellularly, presumably into MVBs. In addition, by studying the mutual requirement of E1 and E2 for the formation of infectious particles, we found that E2 can be incorporated efficiently onto retroviral core particles in the absence of E1, whilst the incorporation of E1 onto HCVpp is strongly dependent on the presence of E2.

## METHODS

**Cells and expression constructs.** 293T human embryo kidney cells (ATCC CRL-1573), COS-7 African green monkey fibroblast kidney cells (ATCC CRL-1651) and Huh-7 human hepatoma carcinoma cells (Nakabayashi *et al.*, 1982) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. MLV-based based green fluorescent protein (GFP)-transfer vector CMV-GFP and GagPol expression vector CMV-GagPol have been described previously (Bartosch *et al.*, 2003b). pHCMV-G, pHCMVcE1E2, pHCMVcE1, pHCMVcE2 and pHCMV-RD114 encode the VSV-G protein, the HCV GPs of genotype 1a as an E1E2 polyprotein or as E1 or E2 individual proteins and the GP of a feline endogenous retrovirus, respectively (Bartosch *et al.*, 2003b). An expression vector encoding the endosomal marker TI-VAMP-GFP (Alberts & Galli, 2003), was co-transfected with *env* and *gag* vectors.

**Antibodies.** The mAb p5D4 (Sigma-Aldrich), against VSV-G, was used diluted to 1:10 000 for Western blotting and to 1:2000 for immunofluorescence (IF) experiments. Anti-RD114 GP (ViroMed Biosafety Labs), a goat antiserum raised against the RD114 gp70 envelope surface protein (SU), was used at 1:3000 for IF experiments. The HCV-E2 GP was detected with mouse H52 at 1:1000 for Western blotting and with undiluted H53 hybridoma supernatant for IF studies. The HCV-E1 GP was detected with mouse A4 at 1:1000 for Western blotting and at 1:500 for IF studies. Anti-MLV capsid (MLV CA; ViroMed Biosafety Labs) is a goat antiserum raised against the Rauscher leukemia virus p30 capsid and was used at 1:10 000 dilution for Western blotting. A rabbit antiserum against MLV capsid p30 (a gift from A. Rein, National Cancer Institute, Frederick, MD, USA) was used at 1:10 000 dilution to identify MLV Gag in IF studies. The secondary Alexa antibodies used for IF were purchased from Molecular Probes.

**Production of HCVpp and infection assays.** Production of HCVpp and infection assays have been described previously (Bartosch *et al.*, 2003b). To analyse the incorporation of HCV envelope GPs into pseudoparticles, HCVpp were pelleted by centrifugation through 20% sucrose cushions and analysed by Western blotting.

**Biotinylation and Western blotting.** Forty hours post-transfection, virion-producer cells were chilled on ice, washed twice with cold PBS (pH 8.0) supplemented with 0.7 mM CaCl<sub>2</sub> and 0.25 mM MgSO<sub>4</sub> (PBS<sup>++</sup>) and incubated with 0.5 mg sulfo-NHS-LC-LC-biotin ml<sup>-1</sup> (Pierce) for 30 min at 4°C. Biotinylation was stopped by incubating the cells with 1 M glycine in PBS<sup>++</sup> for 5 min at 4°C. The cells were then washed with PBS/0.1 M glycine, lysed with



MacDougal buffer [20 mM Tris/HCl (pH 8.0), 120 mM NaCl, 200  $\mu$ M EGTA, 0.2  $\mu$ M NaF, 0.2% sodium deoxycholate, 0.5% Nonidet P-40] containing a protease-inhibitor cocktail (Complete Mini; Roche Diagnostics) and 0.1 M glycine, and centrifuged at 13 000 g for 30 min; 80% of the cell lysates were incubated overnight at 4 °C with streptavidin–Sepharose beads (Pierce). The beads were then washed with MacDougal glycine buffer, resuspended in a denaturing buffer (1%  $\beta$ -mercaptoethanol, 0.5% SDS) and boiled for 5 min. Purified virus samples were obtained by ultracentrifugation of viral supernatants through a 1.5 ml 20% sucrose cushion in a Beckman SW41 rotor (25 000 r.p.m., 2.5 h, 4 °C) and suspended in PBS. All samples were mixed 5:1 (v/v) with a loading buffer [375 mM Tris/HCl (pH 6.8) containing 6% SDS, 30%  $\beta$ -mercaptoethanol, 10% glycerol and 0.06% bromophenol blue], boiled for 5 min and then analysed by SDS-PAGE (12% gel). Western blotting was performed by using standard procedures. SuperSignal West Pico chemiluminescent substrate (Pierce) was used to reveal proteins.

**IF and confocal microscopy imaging.** FuGENE 6 (Roche Diagnostics)-transfected virus-producer cells were grown on 35 mm diameter coverglass dishes coated with D-lysine (Mattek Corporation) or on uncoated 14 mm diameter glass coverslips. IF staining was performed at room temperature 40 h post-transfection. The cells were washed with PBS, fixed for 15 min in 3% paraformaldehyde/PBS, quenched with 50 mM  $\text{NH}_4\text{Cl}$  and permeabilized in 0.2% Triton X-100 for 8 min. Fixed cells were incubated for 1 h with primary antibody in 1% BSA/PBS, washed and stained for 1 h with the corresponding fluorescent, Alexa-conjugated secondary antibody (at 0.5  $\mu\text{g ml}^{-1}$ ) in 1% BSA/PBS. The cells were then washed several times with PBS and mounted on microscope slides with the antifading agent Prolong (Molecular Probes). Images were acquired with an LSM 510 confocal microscope equipped with an Axiovert 100 M microscope (Carl Zeiss) and a  $63\times 1.3$  numerical aperture Apocromat objective. Alexa 488 was excited with an argon laser line at 488 nm and emissions were collected with a band-pass filter (BP505–550). Alexa 546 or 555 was excited, independently of Alexa 488, with a HeNe laser line at 543 nm and emissions were collected with a long-pass filter (LP560).

**EM.** 293T virion-producer cells were harvested 48 h after transfection, pelleted, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed with osmium tetroxide [1% in 0.1 M cacodylate buffer (pH 7.4)]. Cell specimens were dehydrated and embedded in Epon (Epon-812; Fulham). Sections were stained with 7% uranyl acetate in methanol and post-stained with 2.6% alkaline lead citrate in  $\text{H}_2\text{O}$ . Specimens were examined under a JEOL 1200-EX electron microscope and analysed with a MegaView II high-resolution TEM camera and the Soft Imaging system (Eloise). For quantitative EM analysis, at least 50 different cell sections were examined and pseudoparticles were counted in the cytoplasm, in MVBs and at the plasma membrane. For determination of particle size and sphericity index (Gay *et al.*, 1998), a mean diameter for each individual particle was obtained by averaging at least three different diameters measured at 60° angles on the circle delineated by each sectioned particle. To establish the diameter of particles in a given cellular compartment, a minimum of 20 particles were measured.

## RESULTS

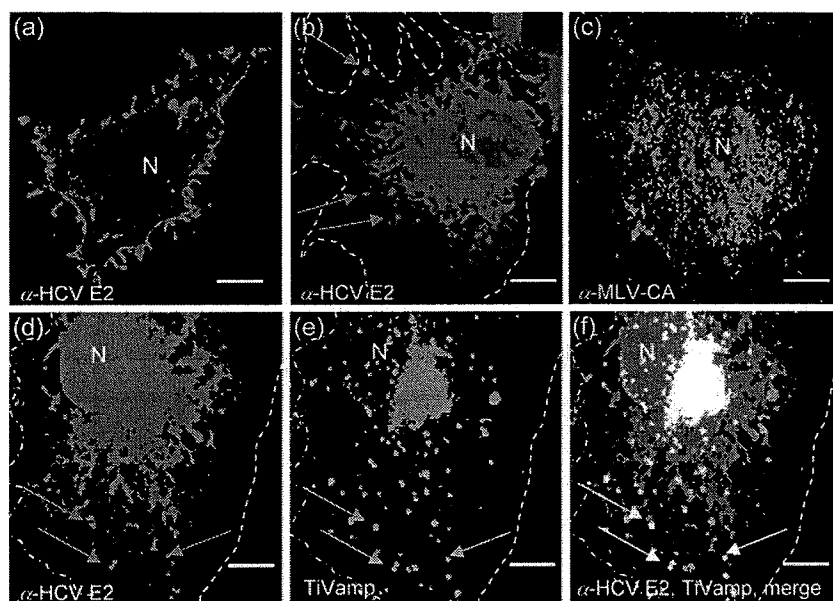
### HCV GPs colocalize with MLV core proteins intracellularly

In cells producing HCVpp, E1 and E2 species are retained mainly in the ER membrane, but, as we have shown

previously by fluorescence-activated cell-sorting (FACS) analysis, a small fraction of these proteins reaches the cell surface (Bartosch *et al.*, 2003b). We asked whether HCV GP species are assembled onto MLV-derived core proteins to form HCVpp at the cell surface or intracellularly. For that purpose, we investigated the colocalization of MLV core proteins with HCV GPs by using IF confocal microscopic analysis. Cell-surface expression of HCV GPs could only be detected in non-permeabilized producer cells (Fig. 1a). In detergent-permeabilized cells, the large amount of intracellularly retained HCV GPs made surface detection impossible, confirming poor cell-surface expression of the HCV GPs (Bartosch *et al.*, 2003b). Interestingly, although intracellularly retained E1 and E2 proteins were concentrated mainly in the ER membranes, they were also observed in some intracellular vesicles (Fig. 1b, d–f and data not shown). This localization pattern of E1 and E2, observed here in cells expressing retroviral core proteins, was also observed in cells expressing the HCV GPs alone (data not shown). Co-staining with cellubrevin, a marker for recycling endosomes (Galli *et al.*, 1994), revealed no colocalization with the HCV GPs. In contrast, co-staining with the TI-VAMP marker (Alberts & Galli, 2003 and references therein) identified some of the HCV GP-containing vesicles as late endosomes and, more particularly, as MVBs (Fig. 1d–f and data not shown). MVBs constitute a cellular compartment that is involved in endocytosis and transport of cargo to lysosomes. However, MVBs also traffic to and fuse with the plasma membrane to release their contents into the extracellular medium (Gould *et al.*, 2003). In contrast to the HCV GPs, MLV core proteins, which form the cores of HCVpp, were not detected in non-permeabilized cells when expressed alone (data not shown). In detergent-permeabilized cells, the MLV core proteins appeared as a punctate and well-dispersed pattern throughout the cytoplasm, with some patches localized under the plasma membrane (Fig. 1c).

It has been shown previously that, depending on the cell type, different retroviral GPs are recruited onto retroviral cores in different cellular compartments (Nydegger *et al.*, 2003; Orenstein *et al.*, 1988; Pelchen-Matthews *et al.*, 2003; Raposo *et al.*, 2002; Sherer *et al.*, 2003). Consistently, as shown by confocal microscopy in Fig. 2, colocalization of GPs of the feline endogenous retrovirus RD114 with MLV core proteins was restricted to intracellular vesicles (Fig. 2a–c) (Sandrin *et al.*, 2004), whilst VSV-G GPs colocalized strongly with retroviral core proteins at the cell surface (Fig. 2d–f) (Guibinga *et al.*, 2004). In colocalization experiments using HCVpp-producing cells, we observed that both E2 and E1 GPs colocalized with MLV core proteins in intracellular vesicles (Fig. 2g–i and j–l), suggesting that assembly may occur intracellularly. The degree of colocalization between HCV GPs and MLV core proteins was less extensive than that observed between RD114 GPs and MLV core proteins. This finding is consistent with the lower infectious titres for HCVpp ( $5\times 10^5\pm 1.2\times 10^5$  IU  $\text{ml}^{-1}$ ) compared with RD114pp ( $7\times 10^6\pm 2.2\times 10^6$  IU  $\text{ml}^{-1}$ ) (Bartosch *et al.*, 2003b). The extent of colocalization of





**Fig. 1.** Localization of viral envelope glycoproteins in HCVpp-producing COS-7 cells by IF. HCVpp-producing cells, expressing the HCV E1 and E2 GPs and MLV core proteins, were untreated (a) or permeabilized (b) and then stained with anti-E2 antibody. (c) Permeabilized cells expressing MLV core proteins stained with an anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles. (d–f) Permeabilized HCVpp-producer cells, which had been co-transfected with the TI-VAMP marker, were stained with anti-E2 antibody (red signal) (d), the TI-VAMP marker (green signal) (e) or both (f). Arrows indicate vesicles where GPs and TI-VAMP colocalize. Outlines of the cell surface and nuclei (N) are indicated. Bars, 10 µm.

HCVpp with MLV core proteins was not dependent on the cell type used for production of HCVpp, as similar observations were made in 293T, COS-7, Huh-7 and HepG2 cells (data not shown).

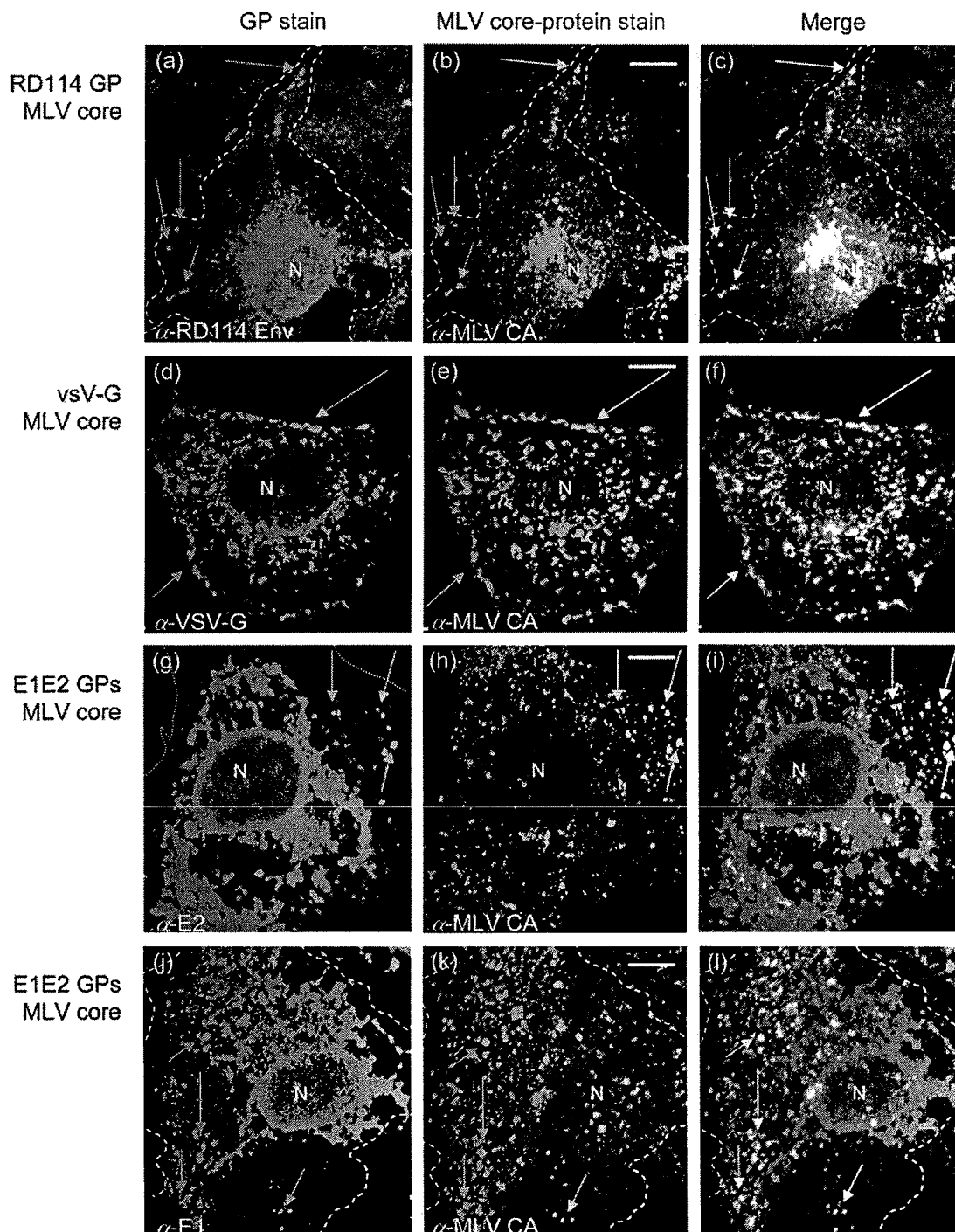
### HCV GPs assemble onto MLV core particles in MVBs

Further investigation of viral-producer cells by EM confirmed our observation that HCVpp formation may occur intracellularly (Fig. 3). By using cells expressing either the MLV core proteins alone or MLV core proteins and VSV-G or HCV GPs, we observed particles within the cytoplasm, in MVBs and at the cell surface (Table 1). Interestingly, the amount of viral particles present at any of these sites within a producer cell differed greatly, depending on which GPs were co-expressed with MLV core proteins. This finding was consistent with previous observations that showed that the viral GPs can influence the localization and assembly pattern of viral particles (Sandrin *et al.*, 2004). In VSV-Gpp-producing cells, particles with a diameter of  $97.31 \pm 19.30$  nm were found exclusively at the cell surface; no particles were detected intracellularly (Table 1). This observation was consistent with our confocal microscopic studies, showing that VSV-G signal colocalized extensively with MLV core proteins at the cell surface (Fig. 2f). Similarly, in cells expressing the MLV core proteins alone, particles were detected mainly at the cell surface (Table 1). The diameter of these particles was  $91.94 \pm 7.7$  nm (Table 1).

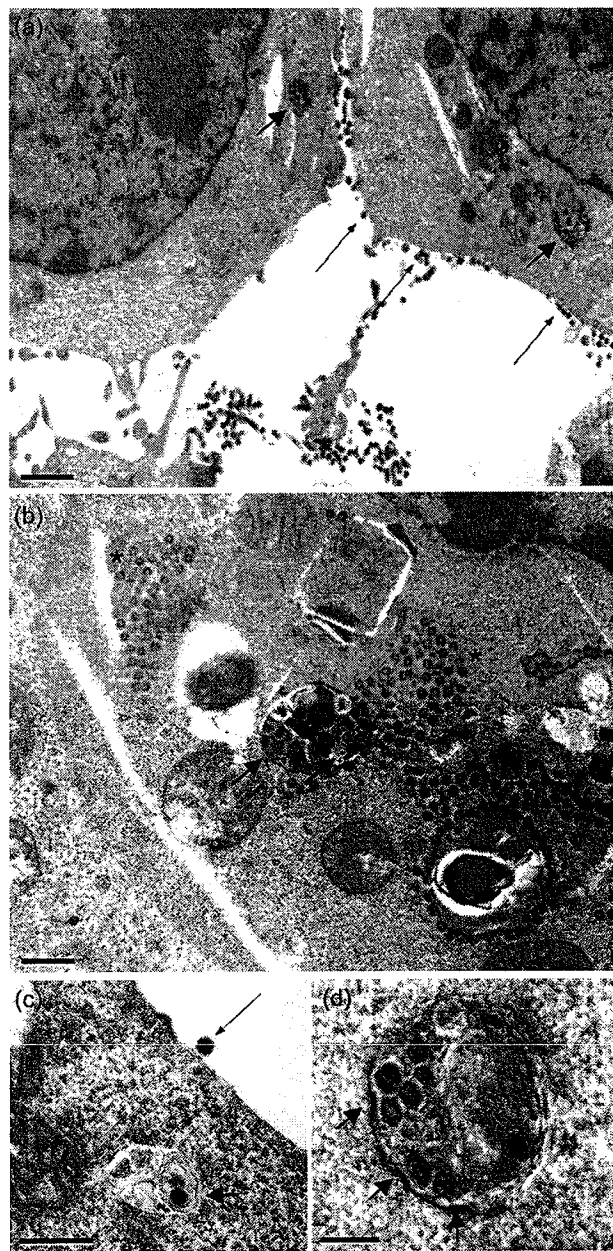
In HCVpp-producer cells, particles were present at the cell surface, but MVBs full of particles were found frequently (Fig. 3a, c). Furthermore, a thickening of the MVB membrane and inwards protrusions suggested that particle budding occurred in this cellular compartment (Fig. 3d) and that particles are secreted into the extracellular medium by fusion of these endocytic vesicles with the cell surface.

When we investigated the size of viral particles released from cells expressing the MLV core proteins alone, the diameter was on average  $91.94 \pm 7.7$  nm (Table 1). In contrast, in HCVpp-producing cells, we found that particles outside the cell, as well as in MVBs, had very similar diameters of  $103.76 \pm 9.26$  and  $103 \pm 10.92$  nm, respectively. Thus, particles present at the cell surface, as well as in MVBs of HCVpp-producer cells, were about 10 % wider in diameter than MLV core particles devoid of viral glycoproteins. This suggests that the HCV GPs can be assembled onto retroviral core proteins within the cell.

Finally, we also observed particles within the cytoplasm of HCVpp-producing cells (Fig. 3b; Table 1). These intracellular particles, however, had a small diameter (approx.  $82.52 \pm 12.74$  nm) when compared with particles produced from cells expressing the MLV core proteins alone ( $91.94 \pm 7.7$  nm) (Table 1). They may represent non-enveloped, cytoplasmic core particles. Cells producing VSV-Gpp did not contain particles within MVBs or the cytoplasm, suggesting that these features are specific to HCVpp-producing cells (Table 1). Thus, to determine which HCV GP induced these phenomena, we investigated by EM the distribution of particles in cells expressing E1 or E2 individually with MLV core proteins. In cells co-expressing E2 and MLV core proteins, particles were observed at the cell surface and in MVBs, but not within the cytoplasm (Table 2). In contrast, in cells co-expressing E1 and MLV core proteins, the proportion of cytoplasmic particles had increased by approximately twofold (from 22 to 41 %; Table 2) compared with cells expressing both E1E2 GPs and MLV core proteins. Furthermore, by using confocal microscopy, whilst we detected some colocalization between MLV core proteins and E2 expressed in the absence of E1 (Fig. 4a–c), we did not detect any colocalization between MLV core proteins and E1 expressed in the absence of E2 (Fig. 4d–f).



**Fig. 2.** Localization of viral envelope glycoproteins and MLV cores in COS-7 cells by IF. COS-7 cells producing RD114pp (a, b, c), VSV-Gpp (d, e, f) or HCVpp (g, h, i, j, k, l) were co-stained with the indicated anti-GP antibodies (a, d, g, j) as well as the anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles (b, e, h, k). Merged images of GP and capsid stains are shown (c, f, i, l). Arrows indicate cell surface or intracellular vesicles where GPs and MLV core proteins colocalize. Comparable results were obtained by using 293T cells. Outlines of the cell surface and nuclei (N) are indicated. Bars, 10 μm.



**Fig. 3.** EM studies of HCVpp-producing 293T cells. Transmission EM of 293T cells expressing the HCV E1 and E2 GPs and MLV core proteins. Several sites and patterns of viral assembly were observed: particles at the cell surface (long arrows), particles within MVBs (thick arrows) and cytoplasmic particles (asterisks). Note a thickening of the MVB membranes and inward protrusions (d), which suggest budding into MVBs. Bars, 1  $\mu$ m (a); 500 nm (b, c); 250 nm (d).

Altogether, these data suggested that E2 colocalizes with MLV core proteins, is incorporated efficiently on particles and allows efficient particle egress. In contrast, E1 colocalizes and assembles efficiently onto MLV core proteins only in the presence of E2.

### Intracellular forms of E2 are incorporated preferentially onto MLV core particles

To confirm and extend our microscopic observations on intracellular HCVpp assembly, we performed biotinylation studies of E1 and E2. Comparison of the amounts and electrophoretic mobilities of biotinylated E1 and E2 expressed at the cell surface to E1 and E2 present on viral particles or in total cell lysates by immunoblotting is shown in Fig. 5. Specificity of biotinylation for cell surface-expressed proteins was controlled by detection of the retroviral core proteins, which are protected from biotinylation by either cell or viral membranes (Fig. 5, bottom panels). Examination of the electrophoretic mobility of E2 revealed different isoforms on viral particles (Fig. 5b, right panel). Importantly, the E2 species found at the cell surface of HCVpp-producing cells migrated much faster in denaturing reducing SDS-PAGE than E2 species incorporated on virions. Because virion-associated E2 species are rather heterogeneous and sensitive to peptide:N-glycosidase F digestion (data not shown) (Op De Beeck *et al.*, 2004) whilst cell surface-expressed E2 species migrate with much higher mobility, E2 proteins must be incorporated into viral particles intracellularly at a stage before the trimming process is complete. After incorporation into viral particles, E2 must be protected from further trimming, whereas unincorporated, monomeric E2 protein is subject to further trimming before it finally reaches the cell surface. These biochemical data, suggesting intracellular recruitment of E2, are therefore fully consistent with our microscopic observations. In contrast, no clear variation of the electrophoretic mobility of E1 incorporated onto viral particles was observed when compared to E1 monomer in total cell lysate or on the cell surface of HCVpp-producing cells (Fig. 5b, left panel).

### E1 requires the presence of E2 for efficient viral incorporation

The prebudding form of the HCV GPs has been described previously to be an E1E2 heterodimer (Deleersnyder *et al.*, 1997; Dubuisson, 2000; Op De Beeck *et al.*, 2001), supporting the notion that E1 should be recruited and assembled within the same intracellular compartment as E2. The fact that E1, expressed in the absence of E2, does not colocalize with MLV core proteins (Fig. 4d–f) indicates that E1 requires the presence of E2 for proper viral incorporation. To compare the incorporation of E1 in the presence or absence of E2, we co-transfected 293T producer cells with equal amounts of E1 and E2 expression constructs either separately or in combination, together with the MLV core and GFP-transfer vectors (Fig. 6). Consistent with previous data (Bartosch *et al.*, 2003b), E2 was incorporated efficiently onto particles both in the absence and the presence of E1 (Fig. 6a). However, in the absence of E1, E2 associated with pseudoparticles migrated with reduced mobility on SDS-PAGE, suggesting an altered glycosylation pattern of E2 in the absence of E1 (Fig. 6a). Pseudoparticle incorporation of E1 in the absence of E2 was detectable, but appeared to be

**Table 1.** Mean diameters of viral particles assembled at different cellular localizations in the presence of various viral envelope glycoproteins

Data are viral particle mean diameters  $\pm$  SD (nm). Viral particles were counted in the cytoplasm, in MVBs and at the plasma membrane. Particles in the process of budding and egressing were included in the counting. Mean diameters of particles were determined as described in Methods. The number of particles analysed (*n*) is shown in parentheses. ND, Not detected in 50 different cell sections.

Envelope	Cellular localization		
	Plasma membrane	MVB	Cytoplasm
None	91.94 $\pm$ 7.70 ( <i>n</i> =43)	ND	ND
VSV-G	97.31 $\pm$ 19.30 ( <i>n</i> =50)	ND	ND
E1 + E2	103.76 $\pm$ 9.26 ( <i>n</i> =23)	103.51 $\pm$ 10.92 ( <i>n</i> =24)	82.52 $\pm$ 12.74 ( <i>n</i> =28)

inefficient, whilst the co-expression of E2 strongly enhanced pseudoparticle incorporation of E1 (Fig. 6a, right panel). Co-expression of both GPs led to the assembly of HCVpp with a titre of about  $10^5$  IU ml<sup>-1</sup> on Huh-7 cells, whilst pseudoparticles incorporating only E1 or only E2 had almost 1000-fold-reduced titres (Fig. 6d, left panel), confirming that both GPs are required to render pseudoparticles infectious (Bartosch *et al.*, 2003b).

To quantify the E2 dependence of E1 for its efficient incorporation and formation of infectious pseudoparticles, we transfected 293T cells with various ratios of E1- and E2-expression plasmids. By co-transfecting increasing amounts of pHCMV-E1 expression plasmid together with steady amounts of pHCMV-E2, we found the expression levels of E2 on virions to be the same in all conditions, confirming that E2 incorporation does not depend on E1 (Fig. 6b). However, when transfecting steady amounts of pHCMV-E1 with increasing amounts of pHCMV-E2, the incorporation levels of both GPs on pseudoparticles increased concomitantly (Fig. 6c). Thus, particle incorporation of E1 depends on the presence of E2 in a dose-dependent manner. In addition, whilst neither E1 nor E2 can form infectious pseudoparticles in the absence of their respective partners,

particle infectivity increases concomitantly with increasing incorporation of both E1 and E2 (Fig. 6d, middle and right panels).

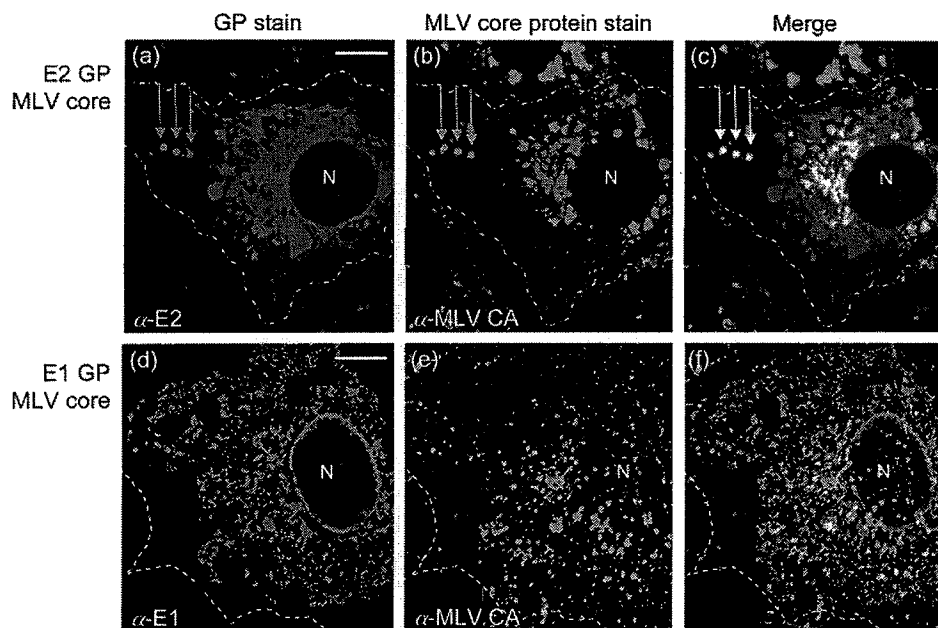
## DISCUSSION

The assembly of wild-type HCV is thought to occur intracellularly (Egger *et al.*, 2002; Greive *et al.*, 2002; Shimizu *et al.*, 1996), in analogy to other flaviviruses and because the structural HCV proteins have been described to be strictly retained in the ER or an ER-derived compartment (Dubuisson, 2000; Egger *et al.*, 2002). Indeed, the transmembrane domains of E1 and E2 contain strong ER-retention signals (Cocquerel *et al.*, 1998, 1999; Flint & McKeating, 1999) and the extremely short cytoplasmic tails of E1 and E2 display no canonical intracellular-trafficking signals. However, upon plasmid-based expression of E1 and E2 in HCVpp-producing cells, a fraction of the GPs trafficks from the ER to the cell surface (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Dumonceaux *et al.*, 2003; Hsu *et al.*, 2003). This suggested that these surface-localized GPs were incorporated onto pseudoparticles (Flint *et al.*, 1999; Lagging *et al.*, 1998; Matsuura *et al.*, 2001; Takikawa *et al.*, 2000). However, in the present work, we showed that, in HCVpp-producing cells, besides localization at the cell surface, HCV GPs are also present in intracellular vesicles, including MVBs, a late endosomal compartment that can traffic and fuse with the cell surface. Moreover, colocalization of the HCV GPs with retroviral core proteins was restricted to intracellular vesicles, including MVBs. This finding is supported by our EM studies, which show that, in HCVpp-producing cells, MVBs contained many viral particles. Furthermore, inward protrusions and thickening of MVB membranes indicated particle formation and budding within this cellular compartment. In addition, the diameters of particles in MVBs of HCVpp-producing cells suggested that the particles contain viral GPs. Yet, the most conclusive evidence suggesting that assembly of HCVpp occurs mainly intracellularly is the results of our biotinylation experiments. In HCVpp-producing cells, surface-expressed forms of E2 do not seem to be incorporated on pseudoparticles because their glycosylation pattern is

**Table 2.** Cellular distribution of viral particles assembled in the presence of the indicated HCV envelope glycoproteins

Data are percentages of VP in cellular compartments. Viral particles were counted in the cytoplasm, in MVBs and at the plasma membrane. Particles in the process of budding and egressing were included in the counting. The number of particles (*n*) analysed in each cellular compartment is shown in parentheses. ND, Not detected in 50 different cell sections.

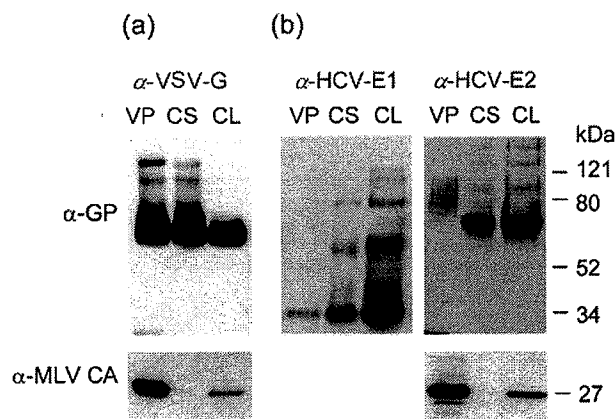
Envelope	Cellular localization		
	Plasma membrane	MVB	Cytoplasm
E1 + E2	48 % ( <i>n</i> =23)	29 % ( <i>n</i> =24)	22 % ( <i>n</i> =28)
E1	47 % ( <i>n</i> =83)	11 % ( <i>n</i> =20)	41 % ( <i>n</i> =71)
E2	90 % ( <i>n</i> =80)	10 % ( <i>n</i> =22)	≤1 % (ND)



**Fig. 4.** Confocal microscopy of HCVpp-producing cells. COS-7 cells co-expressing E2 (in the absence of E1) with MLV core proteins (a, b, c) or co-expressing E1 (in the absence of E2) and MLV core proteins (d, e, f), were co-stained with the indicated anti-GP antibodies (a, d) as well as an anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles (b, e). Merged images of anti-envelope and capsid stains are shown (c, f). Arrows indicate vesicles where envelopes and capsid proteins colocalize. Bars, 10  $\mu$ m.

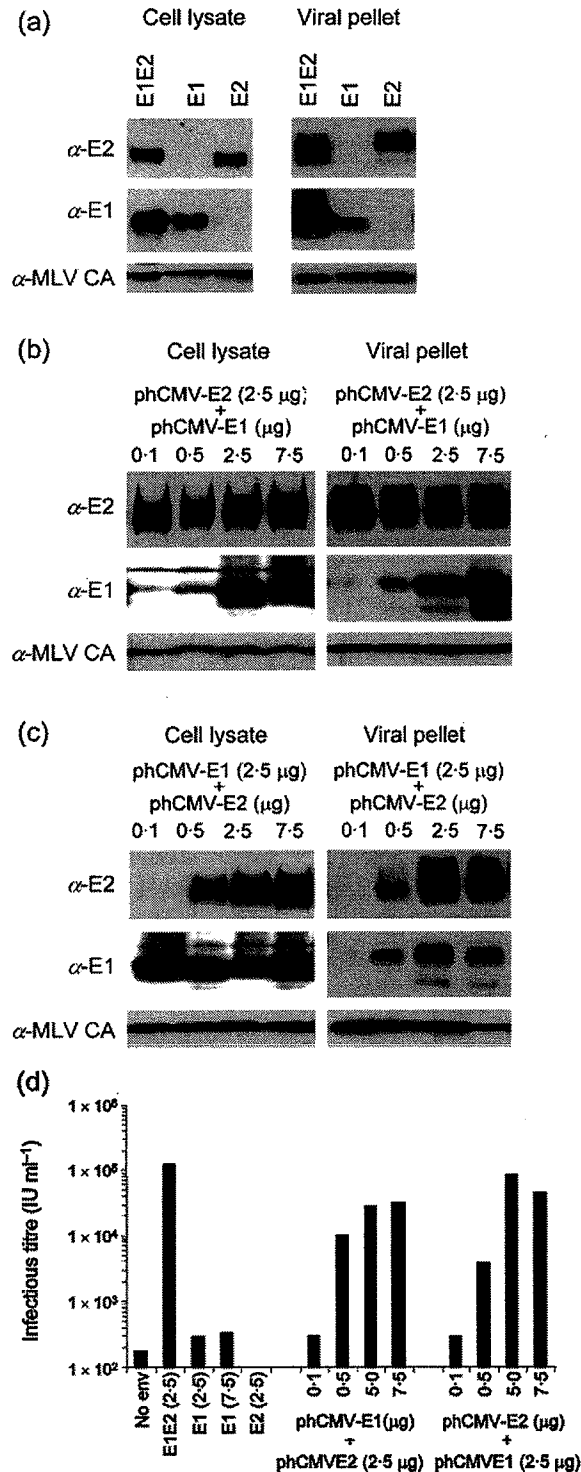
distinct from that of E2 species present on HCVpp, as revealed by their different electrophoretic mobilities. In summary, microscopic and biochemical data show that HCVpp assembly occurs in intracellular vesicles, including

MVBs. This result is consistent with a growing body of evidence suggesting that the assembly of a large number of viruses, including retroviruses and filoviruses, takes place in MVBs (reviewed by Pornillos *et al.*, 2002).



**Fig. 5.** Relative amounts of viral glycoproteins in producer cells and on virions. Viral GPs, VSV-G (a) or both E1E2 GPs (b) and MLV core proteins present in crude cell lysate (CL), at the cell surface (CS) and on sucrose cushion-purified viral particles (VP) were detected by immunoblotting with the indicated antibodies in VSV-Gpp (a)- or HCVpp (b)-producing cells. The cell surface-expressed proteins were biotinylated to allow their purification (see Methods for details).

Determination of structure and assembly of wild-type HCV *in vivo* remains a challenging issue. Reported data based on HCV viral-like particles and HCV replicons suggest that replication occurs in ER-derived compartments and HCV budding may be driven by the core protein (Baumert *et al.*, 1998; Blanchard *et al.*, 2002, 2003; Egger *et al.*, 2002). However, the cellular site of wild-type HCV assembly has remained elusive so far and the close association of HCV biology with lipoprotein metabolism further complicates current views on HCV morphogenesis (André *et al.*, 2005). Previous EM studies on HCV structure have shown that the virus measures 50–60 nm in diameter (Kaito *et al.*, 1994; Shimizu *et al.*, 1996). Wild-type HCV is thus considerably smaller than HCVpp (which has a diameter of 104 nm), a size difference that is probably due to the core proteins. MLV core particles devoid of viral GPs measure 92 nm, whilst the size of HCV nucleocapsid-like particles has been reported in the range 38–62 nm (André *et al.*, 2005). Regarding the assembly of wild-type HCV, Shimizu *et al.* (1996) have reported the detection of enveloped particles in cytoplasmic vesicles of HCV-replicating cells, which suggested that the morphogenesis of wild-type HCV may be vesicle-orientated. Interestingly, they detected HCV in cytoplasmic vesicles that potentially resemble MVBs. With



the very recent development of systems that support wild-type HCV production *in vitro* (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005), it will be interesting to see whether an involvement of MVBs in wild-type HCV morphogenesis can be confirmed.

The respective roles of E1 and E2 in particle assembly were clarified in this study by investigating the expression

**Fig. 6.** Role of E2 in pseudoparticle incorporation of E1. (a–c) Immunoblots of HCVpp. Pseudotyped viruses were pelleted through 20% sucrose cushions. Cell lysates and virus pellets were analysed by SDS-PAGE in reducing and denaturing conditions and revealed with mAbs against E1 and E2 or with an anti-MLV capsid (MLV CA) serum. (a) Immunoblots of HCV pseudotyped particles generated by transfection of  $1.8 \times 10^6$  cells with 2.5  $\mu$ g of either E1 or E2 GP expression construct and 8  $\mu$ g MLV core protein expression construct and 8  $\mu$ g MLV GFP-transfer vector expression construct. phCMV-E1 and phCMV-E2 plasmids were co-expressed or expressed individually. Comparable results were obtained by using COS-7 cells for HCVpp production. (b) Immunoblots of HCV pseudotyped particles generated by co-expression of 2.5  $\mu$ g phCMV-E2 with the indicated amounts of phCMV-E1 ( $\mu$ g). (c) Immunoblots of HCV pseudotyped particles generated by co-expression of 2.5  $\mu$ g phCMV-E1 with the indicated amounts of phCMV-E2 ( $\mu$ g). (d) Infectivity of the generated HCVpp. Titres of corresponding viral supernatants were determined on Huh-7 target cells and are expressed as IU ml<sup>-1</sup>.

patterns of the HCV GPs E1 and/or E2 expressed individually with MLV core proteins. In cells expressing E2 with MLV core proteins, most particles were observed at the cell surface, suggesting efficient particle assembly and egress. In contrast, in cells co-expressing E1 with MLV core proteins, an accumulation of non-enveloped particles in the cytoplasm was observed, suggesting that E1 may possibly inhibit particle assembly and egress by an unknown mechanism. Because the inhibition of E1 on particle assembly and/or egress can be overcome by co-expression of E2, and because E1 colocalizes efficiently with retroviral core only in the presence of E2, our findings suggest that E1 incorporation onto pseudoparticles occurs subsequent to E1E2 heterodimerization. This finding is consistent with previous studies, which showed that the prebudding form of E1E2 is a heterodimer (Cocquerel *et al.*, 2000; Michalak *et al.*, 1997; Op De Beeck *et al.*, 2000, 2004; Patel *et al.*, 2001). Furthermore, we found a direct correlation between the presence of both E1 and E2 and the infectivity of HCVpp, indicating that heterodimer formation and functionality of E1 and E2 are tightly linked processes.

In conclusion, the assembly of HCV GPs on pseudoparticles may be more similar to that of wild-type HCV than currently thought. The morphogenesis of HCVpp requires both HCV GPs and does not occur at the cell surface, but rather is 'vesicle-orientated' and leads to the formation of functional, fusogenic HCV GP complexes on the virion surface.

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